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Human cytomegalovirus infection of THP-1 derived macrophages reveals strain-specific regulation of actin dynamics

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ABSTRACT

Human cytomegalovirus (HCMV) remains latent in cells of the myeloid lineage after primary infection. The THP-1 monocytic cell line is conditionally permissive for infection and has been used primarily to study the process of HCMV reactivation when the cells are induced to differentiate. In the present report, we characterized lytic infection in THP-1 derived macrophages using two strains of HCMV, Towne and BAC-derived TR. Our findings indicate that these cells express viral genes of all three kinetic classes and produce extracellular virus, but that there is a delay in these processes relative to productively infected fibroblasts. Importantly, our studies in THP-1 derived macrophages revealed strain-specific differences in pp65 trafficking and actin dynamics. Based on these observations, our studies indicate that differentiated THP-1 cells can serve as a valuable model for lytic infection.

Introduction

Human cytomegalovirus is a large beta-herpesvirus that is a common, opportunistic pathogen. Infections are generally asymptomatic but the virus remains latent in the host after primary infection. While the virus replicates in numerous cell types, the reservoir for the virus in the body appears to be cells of the myeloid lineage. Work by Soderberg-Naucler and coworkers first identified peripheral blood mononuclear cells as the reservoir of latent virus (Soderberg-Naucler et al., 1997). A number of more recent studies have aimed at identifying the earliest cellular developmental stage that contains latent viral genomes. HCMV has been shown to establish latency in bone marrow CD34+ myeloid progenitor cells (for review, see (Reeves and Sinclair, 2008)). Upon terminal differentiation into macrophages or dendritic cells, the latent virus is reactivated and replicates.

Cells of mononuclear phagocyte system are important in both innate and acquired immune responses to infection (for review, see (Martinez et al., 2006; Murray and Wynn, 2011)). The system is comprised of myeloid progenitor cells in the bone marrow, circulating monocytes, tissue macrophages and dendritic cells distributed throughout the body. In addition to providing the first line of defense in the clearance of microbes by phagocytosis, macrophages and dendritic cells present peptide antigens that prime the acquired immune response. Monocyte differentiation into macrophages is driven by GM-CSF, M-CSF, and CSF-1. The factors activate pro-survival pathways and induce changes in gene expression driving the morphological changes associated with the macrophage phenotype. Primary monocytes isolated from peripheral blood can be used to investigate the process of differentiation in culture; however, because these cells are short-lived, in vitro studies necessitate routine isolation of the cells. The study of differentiation has been greatly facilitated by the availability of human myelomonocytic cell lines such as U937, HL60, and THP-1 that can be induced to differentiate into macrophage-like cells with GM-CSF, M-CSF, retinoic acid, or phorbol esters.

We have chosen to work with THP-1 monocytic cells that lack a functional p53 (Akashi et al., 1999). Numerous studies have utilized THP-1 cells to investigate the process of monocyte differentiation setting the precedent for our use of this cell line (Auwerx, 1991; Kohro et al., 2004). Upon treatment with phorbol esters, THP-1 monocytes develop macrophage-like features both morphologically and functionally. THP-1 cells have previously been described as conditionally permissive for HCMV infection and IE gene expression can be induced after differentiation of the cells. Most studies have used the cells to study viral reactivation (Ioudinkova et al., 2006; Keyes et al., 2012; Saffert and Kalejta, 2010; Sinclair et al., 1992; Yeeet al., 2007); however, Turtinen and Seufzer investigated lytic infection in these cells and showed that the THP-1 derived macrophages supported replication of the laboratory strain Towne, but not AD169 (Turtinen and Seufzer, 1994). A recent report by McCormick and colleagues has extended the characterization of THP-1 derived macrophages during HCMV

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lytic infection (McCormick et al., 2010). In their studies, the authors used THP-1 cells to investigate the role of UL36 in controlling apoptotic pathways during infection. While these authors focused on the effects of infection on cellular pathways, we directed our efforts toward characterizing stages of the viral lytic cycle in THP-1 derived macrophages. Our data indicate that there is a delay in both peak extracellular virus production and virus-induced cell death in THP-1 macrophages relative to the kinetics observed during infection of human fibroblasts at a high multiplicity. Viral proteins of all three kinetic classes are expressed within 4 days post-infection in the macrophage-like cells but viral DNA replication is slow to increase within this time period. Comparison of two HCMV strains, Towne and BAC-derived TR (TR-BAC), did not reveal substantial differences in titers, viral DNA replication, or viral gene expression. Interestingly, we observed a significant delay in the cytoplasmic accumulation of pp65 in THP-1 derived macrophages. This delay was especially pronounced in cells infected with the BAC-derived TR strain of HCMV and this phenotype was later also detected in fibroblasts infected with this strain. In addition to the differences observed in pp65 localization, we noted that the morphology of THP-1 macrophages infected with the two strains of HCMV was strikingly different. This observation led us to investigate the distribution of actin filaments in cells infected with either strain. Our findings indicate that Towne and TR-BAC infections had distinct effects on the actin cytoskeleton, such that TR-BAC strongly induced assembly of actin stress fibers. These filaments were also evident in Towne-infected THP-1 macrophages, but at a lower frequency. In contrast, Towne-infection of primary fibroblasts led to depolymerization of actin while stress fibers were maintained during TR-BAC infection. Despite these cell type-specific differences in Towne-induced remodeling of the actin cytoskeleton, the studies presented here validate the use of THP-1 derived macrophages as a model for lytic infection.

Results

HCMV efficiently infects THP-1 derived macrophages but extracellular virus production is delayed

For our initial characterization of HCMV infection of differentiated THP-1 cells, the efficiency of infection and production of extracellular virus were determined. THP-1 cells were treated with PMA one day prior to infection to induce differentiation. Twenty-two to 24 h later, the adherent cells were infected at an MOI of 5 with either Towne or TR-BAC in the presence of phorbol myristate acetate (PMA) and hydrocortisone (HC). The inoculum was removed 24 h later and the cultures were fed with media containing PMA and HC. At 48 h p.i., the efficiency of infection was determined by staining coverslips with antibodies to the immediate early proteins (IE1-72 and IE2-86) and the DNA polymerase accessory protein (UL44). In spite of infecting cells at a multiplicity of 5, the percentage of IE-positive (IE+) cells did not approach 100%; in fact, the percentage of IE+ cells varied and ranged between 68 and 86% over a series of experiments (Table 1). The difference between percentages of infected cells (IE positivity) for the two strains within a given experiment was generally within 10%. We also determined the ratio of UL44+/IE+ cells to investigate whether there were strain-specific differences in the transition from IE to E gene expression in the infected THP-1 derived macrophages. As shown in Table 1, we did not observe any strain-specific differences in the transition to early gene expression as determined by UL44 expression. In some experiments the UL44+/IE+ ratio was higher in Towne infected cultures, while it was higher in TR-BAC infected cultures in other experiments.

Table 1
Percentage HCMV-infected THP-1 derived macrophages at 48 h p.i.

Expt.	Virus	%IE+	%UL44+	%UL44+/IE+
1	Towne	70	63	90
	TR-BAC	72	71	99
2	Towne	77	67	87
	TR-BAC	86	75	87
3	Towne	74	71	96
	TR-BA	85	68	81
4	Towne	86	73	85
	TR-BAC	86	70	81
5	Towne	75	60	79
	TR-BAC	68	57	84

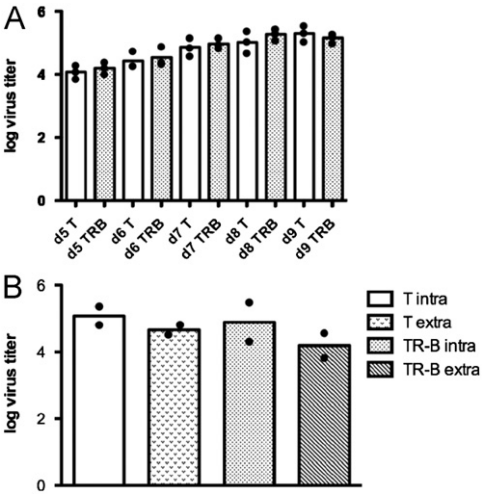

Fig. 1. Extracellular virus production by THP-1 derived macrophages. (A.) THP-1 cells were treated with PMA to induce differentiation for 22–24 h prior to infection with HCMV Towne (T) or BAC-derived TR (TRB) at a multiplicity of 5 as described in the Materials and Methods. At the time points indicated, supernatants were collected and frozen. Viral titers were determined by plaque assay using primary human fibroblasts. Bars represent the mean of values from three independent experiments indicated by the scatter plot. (B.) Differentiated THP-1 cells were infected as described in the Materials and Methods. At d7 p.i., supernatants and cells were collected. Cells were lysed to release pools of intracellular virus that were measured by plaque assay as above. Bars represent the mean of values from two independent experiments indicated by the scatter plot.

We then compared the production of extracellular virus in differentiated THP1 cells infected with either Towne or TR-BAC. For these experiments, we examined the accumulation of virus in cultures from d 5 to d 9 p.i., without replenishing PMA/HC in the culture media. Supernatants were collected until the majority of cells had lifted from the bottom of the tissue culture vessel. The kinetics of extracellular virus production are shown in Fig. 1A. Not unexpectedly, the production of virus increased over time and titer approximately doubled every 24 h; however, we did not observe differences in extracellular virus production between Towne and TR-BAC samples. Consequently, we measured intracellular virus production in THP-1 derived macrophages infected with either Towne or TR-BAC to test whether there was a difference between the strains in this cell type. Towne and TR-BAC infected cells contained comparable quantities of intracellular virus titers (Fig. 1B). An unexpected observation was the survival of the THP-1 derived macrophage-like cells for greater than 7 d after infection at a high MOI; however, the extended period of viability and production extracellular virus is similar to that previously described for primary monocyte-derived

macrophages (Frascaroli et al., 2009; Sinzger et al., 2006) and THP-1 derived macrophages (McCormick et al., 2010).

Viral DNA accumulates slowly in THP-1 derived macrophages

In addition to analyzing trends in virus production, the kinetics of viral DNA replication between strains was compared. THP-1 cells were differentiated as before and infected at high MOI with either Towne or TR-BAC. At 24 h p.i., the inoculum was removed and cells were collected by trypsinization. Cell pellets were washed before they were frozen. The quantity of viral DNA at subsequent time points was measured relative to the level in this 24 h sample. At 24 h intervals, cells were harvested and pellets were stored until the end of the experiment. DNA was extracted from cells as previously described (Sanchez and Spector, 2006; White et al., 2004). Viral DNA was measured by real-time PCR using primers and probe specific for the UL77 gene. DNA samples were normalized to the cellular beta-actin gene using commercially available primers and probe. Fig. 2A shows the kinetics of viral DNA replication in THP1-infected cells in three independent experiments. It should be noted that at 24 h p.i., THP-1 macrophages infected with TR-BAC contained 2–3 fold more genomes than cells infected with Towne, most likely due to differences in the particle-to-PFU ratios of the stocks used to infect the cultures. In general, the viral DNA increased less than 10-fold within 72 h p.i. relative to the quantity of viral DNA at 24 h p.i., with the exception of a single experiment with TR-BAC. The slopes of the curves became steeper after 4 d p.i. The accumulation of viral DNA in THP-1 derived macrophages was slow at early times relative to similar experiments in human fibroblasts (Fig. 2B). In fibroblasts infected with the Towne strain, there is an approximately 15-fold increase in viral DNA between 24 and 48 h p.i. Thus we observe a significant delay in viral DNA replication in the THP-1 derived macrophages, which likely contributes to the delay in extracellular virus production.

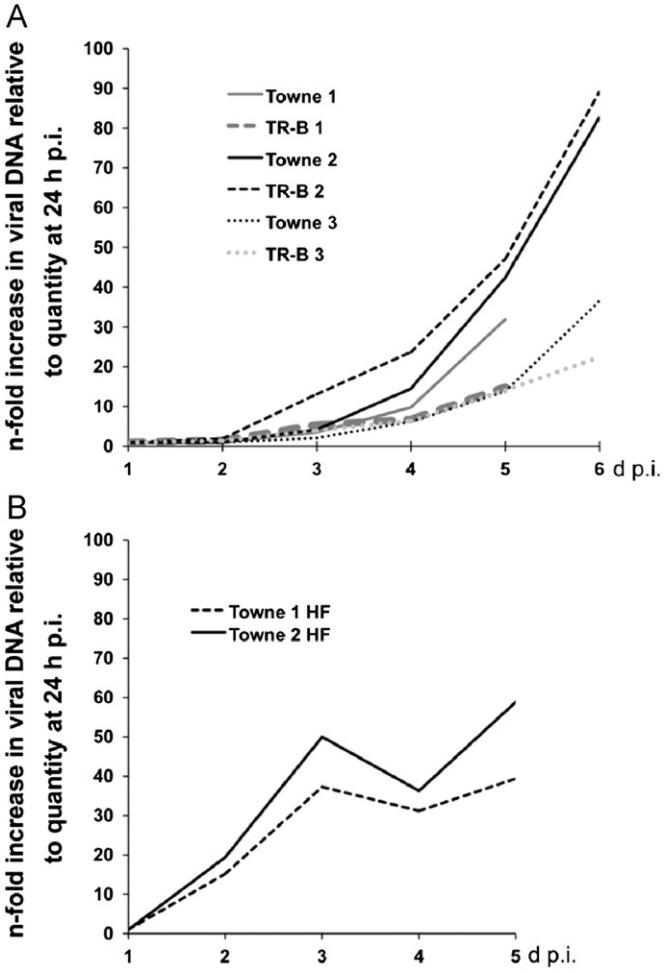

Fig. 2. Viral DNA replication in THP-1 derived macrophages. (A.) THP-1 cells were treated with PMA to induce differentiation for 22–24 h prior to infection with HCMV Towne or BAC-derived TR (TR-B) at a multiplicity of 5 as described in the Materials and Methods. At the time points indicated, infected cells were collected and frozen. DNA was extracted and used as template for RT-PCR with primers to HCMV UL77 and human beta-actin as described in the Materials and Methods. Amplification of viral DNA is shown relative to the quantity present at 24 h p.i. Results from three independent experiments are shown. (B.) For comparison, the kinetics of viral DNA replication in permissive human foreskin fibroblasts (HF) is shown. G_0-synchronized fibroblasts were infected with HCMV Towne at an MOI of 5. Replication was measured as described above. (A) Replication in THP- cells, (B) Replication in human fibroblasts.

The transition to early gene expression is delayed in THP-1 derived macrophages

We next determined the pattern of viral gene expression by immunoblotting for proteins of the three kinetic classes. The results from representative blots are shown in Fig. 3. The IE proteins were detected within 24 h p.i. We observed that the

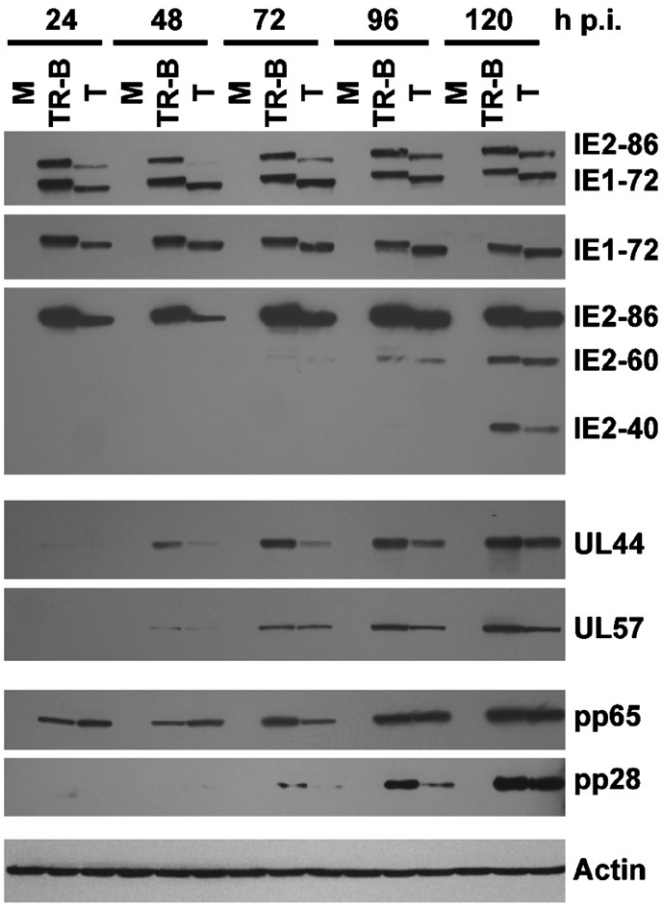

Fig. 3. Viral protein expression in THP-1 derived macrophages. THP-1 cells were treated with PMA to induce differentiation for 22–24 h prior to infection with HCMV Towne (T) or BAC-derived TR (TR-B) at a multiplicity of 5 as described in the Materials and Methods. At the time points indicated, infected cells were collected and frozen. Equivalent cell numbers were loaded into wells of SDS-PAGE gels. Proteins were resolved and transferred to nitrocellulose as previously described. Membranes were probed with antibodies to IE (IE1-72 and IE2-86, -60, and -40), E (UL44, UL57, pp65) and L (pp28) proteins. Antibody to beta-actin was used as a loading control. Mock-infected cells are indicated in lanes labeled M.

migration patterns of both IE1-72 and IE2-86 differed between the two strains of virus. The antibody CH16.0 recognizes the common exon 2 and thus detects both IE proteins (top panel, Fig. 3). We observed a similar shift in size when we probed membranes with antibodies specific for IE1 or IE2 that recognize the unique exon 4 or exon 5 of each protein, respectively. The IE2-specific antibody also detects smaller forms of IE2, denoted p40 and p60. These smaller forms of IE2 also migrate faster in samples from Towne-infected cells (Fig. 3). The elevated steady state levels of IE proteins at early times post-infection in cells infected with TR-BAC likely reflect the 2–3 fold greater quantity of genomes present in the cells. We also noted a change in the IE1/IE2 ratio between Towne and TR-BAC infected cells, suggesting differences in the utilization of IE splice sites within cells infected with either strain (Fig. 3 and Supplementary Table 1).

The expression patterns of the early and early-late proteins UL44, UL57, and pp65, were also examined. We did not detect substantial differences in the expression of these proteins between virus strains at the various time points, except in the rates of accumulation of UL44 (Supplementary Table 1). However, by the late time points the levels of UL44 were comparable between the strains. Similar observations were made for the expression of the true late protein pp28. The slow accumulation of early and late proteins is consistent with the delayed onset of viral DNA replication.

Cytoplasmic localization of the tegument protein pp65 late in infection occurs slowly in THP-1 macrophages

As the next step in characterizing the HCMV lytic cycle in THP-1 macrophages, we analyzed the localization of the abundant tegument protein pp65 (UL83). This protein exhibits a biphasic pattern of localization in infected fibroblasts; at early times post-infection, the protein is nuclear while it is exclusively cytoplasmic at late times (Sanchez et al., 2000, 2007). We observed the biphasic pattern of pp65 localization in infected THP-1 derived macrophages, but there was a delay in the transition of pp65 to the cytoplasm (Fig. 4). In fibroblasts infected with HCMV Towne at a high multiplicity, pp65 is localized to the cytoplasm in the majority of cells at 72 h p.i. (Sanchez et al., 2007; Sanchez et al., 2000). In contrast, in THP-1 macrophages infected with Towne, only 49% contained some pp65 in the cytoplasm at 120 h p.i. (average of 2 independent experiments). At 144 h p.i, pp65 was detected in the cytoplasm of approximately 53% in cells infected with Towne and approximately 20% of cells no longer contained pp65 in their nuclei. Surprisingly, pp65 appeared to be more strongly localized to the nuclei of cells infected with TR-BAC in comparison to Towne, as shown in Fig. 4. At 120 h p.i, approximately 23% of TR-BAC-infected THP-1 macrophages contained detectable levels of pp65 in cytoplasm (average of 2 independent experiments). The percentage of TR-BAC infected cells containing pp65 in the cytoplasm did increase over time but cells that were completely devoid of nuclear pp65 were not detected at 144 h p.i. In order to determine if this was a cell type-specific effect on pp65 localization, we examined pp65 localization in Towne and TR-BAC infected primary human fibroblasts. As was previously described, greater than 95% of cells infected with Towne contained pp65 exclusively in the cytoplasm at 96 h p.i. (Fig. 5, average of two experiments). LAP2 (lamina-associated protein 2) staining was included to demarcate the margins of the nuclei (Dechat et al., 2000). In contrast, 70% of cells infected with TR-BAC contained pp65 in their nuclei at 96 h p.i. (average of 2 experiments), suggesting that the complete evacuation of pp65 from the nucleus that occurs late in infection may not occur with all HCMV strains or occurs with different kinetics.

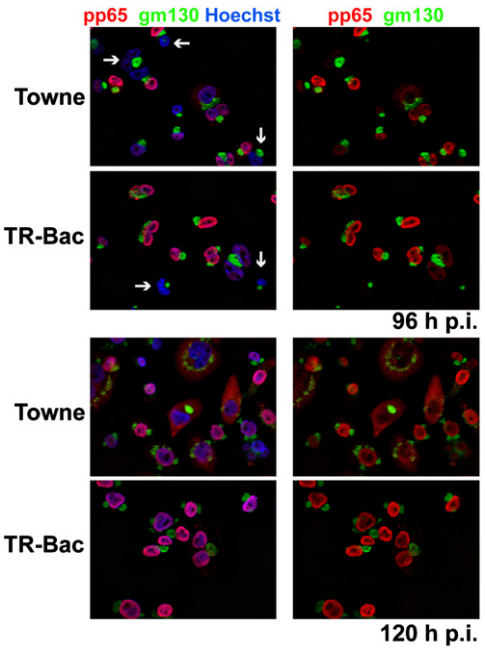

Fig. 4. pp65 localization in THP-1 derived macrophages. THP-1 cells were seeded onto glass coverslips and treated with PMA to induce differentiation. Twenty-four h later, cells were infected with HCMV Towne or BAC-derived TR at a multiplicity of 5 as described in the Materials and Methods. At 96 and 120 h p.i., cells were rinsed with PBS and fixed in 2% paraformaldehyde. Coverslips were stained as previously described with antibodies to HCMV pp65 and the cis-Golgi marker GM130. Hoechst was used as a nuclear counterstain. Arrows indicate uninfected cells.

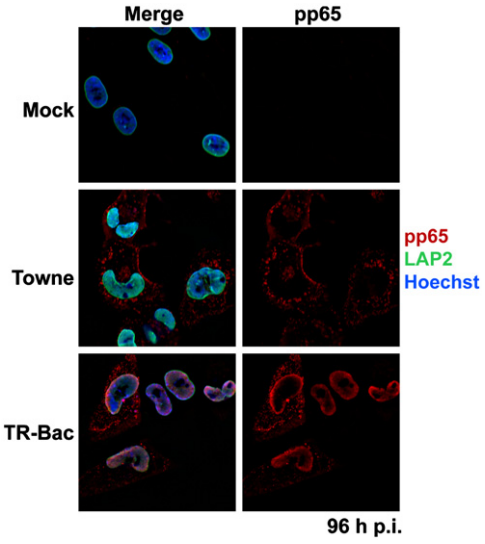

Fig. 5. pp65 localization in primary human fibroblasts. G_0-synchronized fibroblasts were seeded onto glass coverslips and infected with HCMV Towne or BAC-derived TR at an MOI of 5, or mock-infected. At 96 h p.i., cells were rinsed with PBS and fixed in 2% paraformaldehyde. Coverslips were stained as previously described with antibodies to HCMV pp65 and the cellular protein LAP2. Hoechst was used as a nuclear counterstain. Confocal images of 0.5 μM sections were collected along the *Z*-axis but one single plane through the middle of the cells is shown.

Strain-dependent differences in the morphology of infected THP-1 derived macrophages are correlated with changes in the actin cytoskeleton

We consistently observed that infection of THP1-derived macrophages with TR-BAC but not Towne strain caused the cells

to flatten and elongate starting at 48 h p.i. (Fig. 6). This phenotype was not evident in cultures infected with Towne until 96 h p.i., and to a lesser degree. Because our experiments utilized unpurified supernatants instead of gradient purified virus, we investigated if the changes in cell shape were induced by molecules secreted into the supernatants, the viral secretome (Streblow et al., 2008). Towne and TR-BAC stocks were subjected to UV irradiation as previously described (Fortunato et al., 2000) and used to infect THP-1 derived macrophages as in Fig. 6. Interestingly, we did not observe changes in cell morphology in TR-BAC infected cultures when they were infected with UV-irradiated virus (Fig. 7). These results suggest that viral gene expression is required for the induction of the elongated morphology adopted by TR-BAC infected THP1-derived macrophages.

We next investigated the mechanisms that resulted in altered THP-1 cell morphology upon infection. We hypothesized that such effects would be mediated by differences in the organization of the cytoskeleton; therefore, we stained cells infected with either strain with FITC-phalloidin, which binds to F-actin and stains actin filaments. Fewer than 5% of mock-infected cells contained actin filaments (Fig. 8A and Supplementary Figs. 1–3). Instead the phalloidin staining was distributed in a dot-like pattern reminiscent of podosomes (Burger et al., 2011). In contrast, we readily observed the presence of actin filaments in THP-1 derived macrophages infected with TR-BAC (Fig. 8 and Supplementary Figs. 1–3). Cells infected with the Towne strain also contained actin filaments but they were thinner and less abundant in the cells (Fig. 8 and Supplementary Figs. 1–3). We quantified the number of THP-1 derived macrophages containing elongated, parallel actin filament bundles and found that the number increased over time in cultures infected with either strain (Fig. 8B); however, the percentage of TR-BAC infected THP-1 macrophages was greater at all time points analyzed. The greatest difference occurred at 48 h p.i., when there was an approximately 5-fold higher percentage of actin fiber-positive cells in cultures

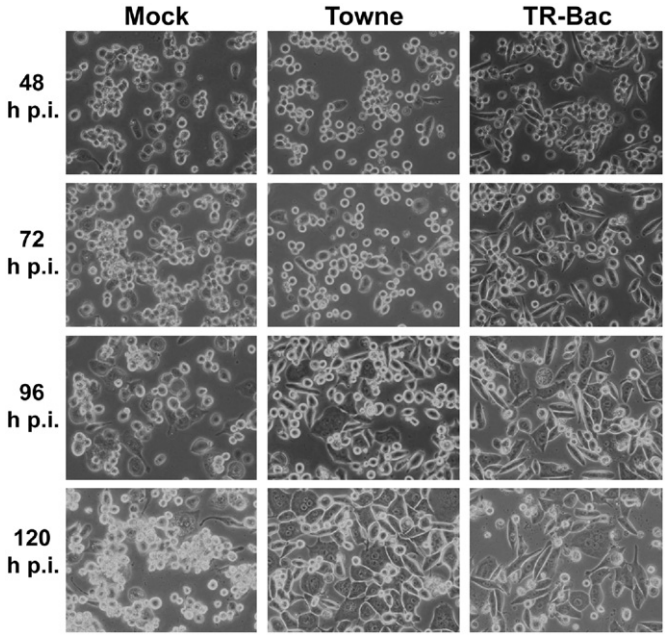

Fig. 6. Morphology of infected THP-1 derived macrophages. THP-1 cells were treated with PMA to induce differentiation for 22–24 h prior to infection with HCMV Towne (T) or BAC-derived TR at a multiplicity of 5 as described in the Materials and Methods. At the time points indicated, phase contrast images of cultures were recorded.

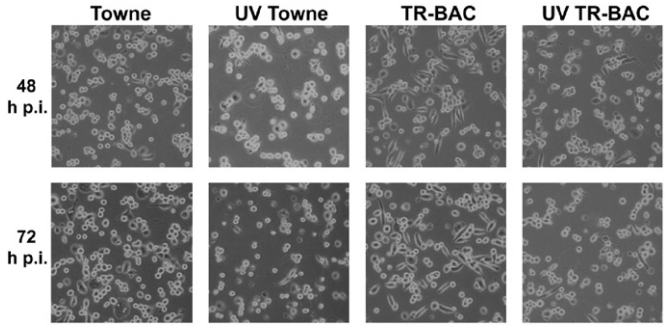

Fig. 7. UV irradiation of TR-BAC inhibits changes in cell morphology upon infection. THP-1 cells were treated with PMA to induce differentiation for 22–24 h prior to infection with UV-irradiated HCMV Towne (UV T) or BAC-derived TR (UV TR-Bac) at a multiplicity of 5 as described in the Materials and Methods. Non-UV treated virus served as controls. At the time points indicated, phase contrast images of cultures were recorded.

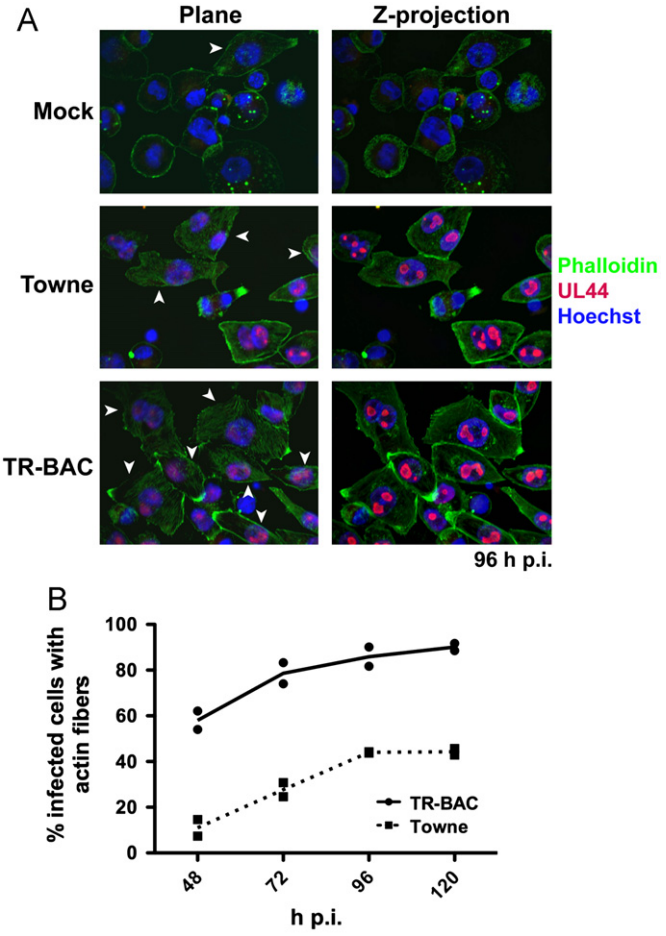

Fig. 8. Organization of the actin cytoskeleton in HCMV-infected THP-1 derived macrophages. THP-1 cells were seeded onto glass coverslips and treated with PMA to induce differentiation. Cells were infected with HCMV Towne or BAC-derived TR at a multiplicity of 5 as described in the Materials and Methods. At the time points indicated, cells were rinsed with PBS and fixed in 2% paraformaldehyde. Coverslips were stained as previously described with antibody to HCMV UL44 and FITC-labeled phalloidin. Hoechst was used as a nuclear counterstain. Confocal images of 0.5 μM sections were collected along the *Z*-axis. (A.) Panels on the left show images of single planes near the bottoms of the cells where they attach to the coverslips. Panels on the right show corresponding Z-stack projections representing merged images of all planes of the field. The white arrowheads indicate the positions of cells with actin fibers. (B.) The percentage of infected cells containing actin filaments was quantified at the time points indicated. Squares and circles indicate the values for Towne- and TR-BAC infected cells, respectively, for two independent experiments. The line connects the mean values for the individual time points.

infected with TR-BAC. Although the percentage increased over time in Towne samples, the peak level (44% at 120 h p.i.) was below than the lowest percentage observed for TR-BAC infections (58% at 48 h p.i.).

The actin phenotypes observed in the THP-1 derived macrophages were puzzling since it was previously shown that HCMV infection of fibroblasts and primary macrophages caused the depolymerization of actin stress fibers (Sanchez et al., 2000; Seo et al., 2011; Sharon-Friling et al., 2006). Therefore, we proceeded to analyze the effects of TR-BAC infection on actin polymerization in primary fibroblasts. Unlike mock-infected THP-1 macrophages (Fig. 8A), mock-infected fibroblasts contained actin stress fibers (Fig. 9E). Surprisingly, we observed that cells infected with TR-BAC contained thick actin stress fibers (Figs. 9C and D); however, as previously reported, primary fibroblasts infected with Towne contained few actin fibers (Figs. 9A and B, and Supplementary Fig. 4). These fibers were thinner and less abundant than those observed in TR-BAC infected fibroblasts, a phenotype also observed in THP-1 derived macrophages. Again we quantified the number of infected cells containing elongated, parallel actin bundles and determined that actin filaments were depolymerized over time in cells infected with Towne (Fig. 9F). In contrast, even at 120 h p.i., greater than 90% of cells infected with TR contained easily discernable actin filaments (Fig. 9F, average of two experiments). Taken together with our observations made in THP-1 derived macrophages, these results suggest that the degree of actin polymerization during infection is both cell type-dependent and HCMV strain dependent.

Discussion

Because HCMV remains latent in cells of the myeloid lineage, numerous studies have investigated infections in monocytes and monocyte-derived macrophages (Chan et al., 2008; Frascaroli et al., 2009; Keyes et al., 2012; Smith et al., 2004; Soderberg-Naucler et al., 1997). Unlike previous studies, we have utilized the THP-1 monocytic cell line as a model for lytic replication in macrophages. THP-1 cells have been used primarily as a model to study HCMV reactivation from latency but our studies indicate that they may also be useful for examining aspects of productive viral infection. One advantage of using these cells is that large numbers of cells can be propagated easily as opposed to isolating primary monocytes from peripheral blood. Secondly, experiments can be conducted within a single genetic background, which may not necessarily be the case when isolating monocytes from donor blood products. The major caveat to using these cells is their lack of functional p53 (Auwerx, 1991), which has been shown to be required for efficient infection in fibroblasts (Casavant et al., 2006); however, our comparative studies presented here indicate that infections in THP1-derived macrophages resemble infections in primary fibroblasts, although we did observe some cell type-specific differences in actin organization. In addition to the results shown in this report, we have previously demonstrated that the ABCA1 transporter is down-regulated primary fibroblasts and THP-1 derived macrophages (Sanchez and Dong, 2010), further validating the use of THP-1 macrophages as a model for infection of primary cells.

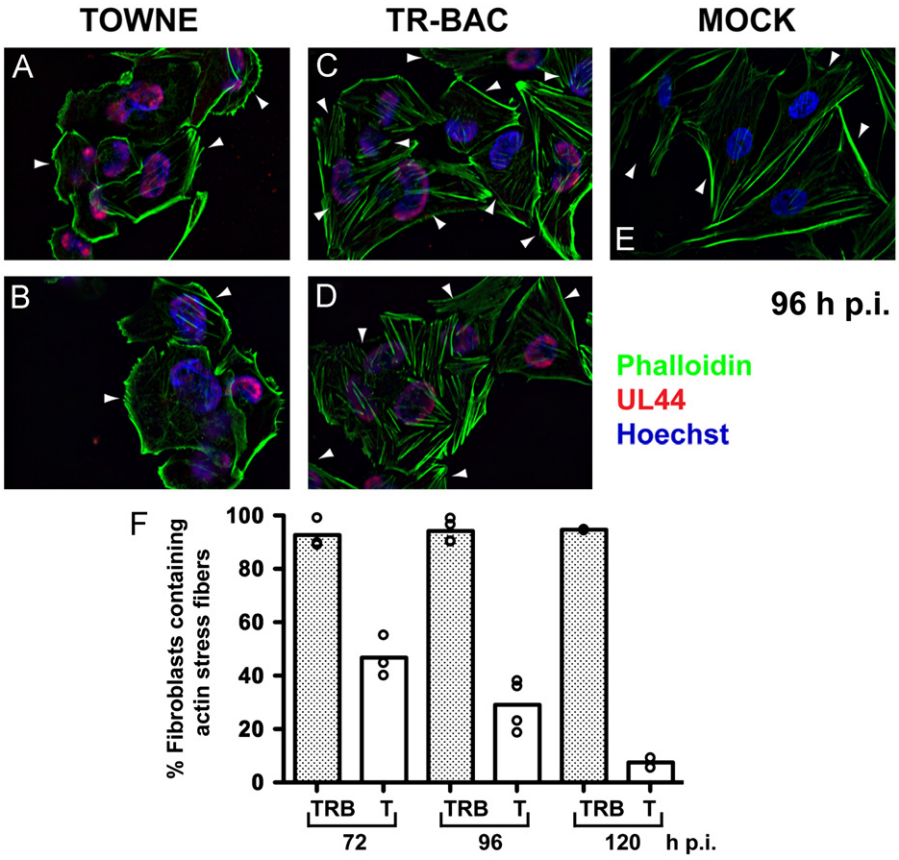

Fig. 9. Organization of the actin cytoskeleton in HCMV-infected human primary foreskin fibroblasts. G_0-synchronized fibroblasts were seeded onto glass coverslips and infected with HCMV Towne or BAC-derived TR at a mulitplicity of 5. At the time points indicated, cells were rinsed with PBS and fixed in 2% paraformaldehyde. Coverslips were stained as previously described with antibody to HCMV UL44 and FITC-labeled phalloidin. Hoechst was used as a nuclear counterstain. (A–E) Panels show images of single planes near the bottoms of the cells where they attach to the coverslips. The white arrowheads indicate the positions of cells with actin fibers. (A, B): Towne-infected human fibroblasts; (C, D): TR-BAC infected human fibroblasts; (E): mock infected fibroblasts. (F.) The percentage of infected cells containing actin filaments was quantified at the time points indicated. Circles indicate the values for Towne- and TR-BAC-infected cells for each independent experiment. Columns indicate the mean value for each time point determined from 2 to 4 independent experiments.

While cell cultures are treated with PMA to induce differentiation one day prior to infection, we observed that a subset of cells did not express IE proteins 48 h p.i., despite infection at high multiplicity. The percentage of cells that did not express IE proteins varied between 15 and 35% over multiple experiments (Table 1), which may reflect differences in the rate of development of the THP-1 monocytes into macrophage-like cells. McCormick and colleagues reported that surface expression of CD11b and CD14, markers of macrophage differentiation, increased by day 3 post differentiation of the THP-1 cells (McCormick et al., 2010). It is possible that altering the protocol for inducing differentiation may increase the efficiency of infection; in fact, it was recently shown that THP-1 cultures that were allowed to rest for 5 days after treatment with phorbol esters to induce differentiation displayed multiple markers and activities that made them indistinguishable from primary macrophages (Daigneault et al., 2010).

As shown in Fig. 2, there was an eclipse period following infection before there was a significant increase in viral DNA replication in THP-1 derived macrophages. It is unclear if the delay in replication also occurs in primary monocyte-derived macrophages or results from the absence of functional p53 in THP-1 cells. Casavant and colleagues reported slow accumulation of viral DNA in p53-negative fibroblasts relative to wild type cells (Casavant et al., 2006). The steady state levels of the early proteins UL44 and UL57 that are necessary for replication remained low until 72 h p.i. in THP-1 macrophages, consistent with the delay in replication. By 120 h p.i., the viral protein levels were similar between samples infected with each strain. At this time point, comparable amounts of virus were also released by cells infected with Towne or TR-BAC. Because TR-BAC is a clinical strain, we expected that it would replicate more efficiently than Towne in the THP-1 derived macrophages but we did not observe a marked difference between the strains. As mentioned above, viral replication is slow in p53-negative fibroblasts (Casavant et al., 2006); therefore, the p53 mutation in THP1 cells may mask any growth advantage that TR-BAC may have. Alternatively, the result that the two strains are similar in terms of extracellular virus production in the THP-1 macrophage model, but not in fibroblasts, is reminiscent of previous observations by Adler and colleagues. It has been shown that the presence of wild type UL131A in clinical strains has an inhibitory effect on virus release in fibroblasts but not in endothelial cells (Adler et al., 2006). We noted that the TR-BAC infected fibroblasts do not produce as much extracellular virus as Towne-infected fibroblasts (data not shown), confirming the defect in virus release from these cells. Thus our results in THP1 derived macrophages may reflect the effects of UL131A on TR-BAC release, as well as the p53 defect in this cell type.

While there were some modest differences in the accumulation of IE proteins in cells infected with Towne and TR-BAC at early times (Fig. 3), the most striking observation was that the IE proteins (full length IE1, IE2, and IE2 p60 and IE2 p40) migrated more slowly in TR-BAC samples. Analysis of UL122–123 sequence in TR-BAC showed a small number of sequence changes in comparison to the Towne strain, which could alter the migration of the proteins. It is also possible that migration was affected by differences in the pattern of post-translational modifications that occur in exons 2, 3, 4, and 5. We also observed that the IE1/2 ratio was different between the two strains at late times (Supplementary Table 1), but software analysis predicted that similar splice sites were present in the sequences. Thus, other factors likely influence splice site-utilization in the UL122-123 locus.

Although we did not observe major differences in the growth characteristics of the laboratory strain Towne versus the clinical strain TR-BAC in THP-1 derived macrophages, some distinct morphological changes were noted in cells infected with TR-BAC. These results were correlated with the presence of actin filaments in TR-BAC-infected THP-1 macrophages at higher frequency than in Towne-infected cells. It has been well documented that infection with AD169, Towne, and Toledo strains results in disruption of the actin cytoskeleton and/or focal adhesions in fibroblasts raising the possibility that we were observing either cell type-specific and/or virus-strain specific differences in actin mobilization in the THP-1 derived macrophages (Sanchez et al., 2000; Seo et al., 2011; Sharon-Friling et al., 2006; Stanton et al., 2007). Additionally, Frascaroli and coworkers reported that the actin cytoskeleton was reorganized in primary macrophages infected with HCMV TB40E but they did not report the presence of actin fibers in infected cells (Frascaroli et al., 2009). Their observations raised the possibility that our results in THP-1 macrophages were an artifact of using a transformed myeloid cell type. However, fibroblasts infected with TR-BAC also contained abundant actin filaments while those infected with Towne strain did not late in infection, indicating that virus strains differ in their ability to remodel the cytoskeleton even in primary cells. We postulate that the difference in the kinetics of actin polymerization between Towne-infected THP-1 macrophages and Towne-infected fibroblasts may reflect the difference in the initial state of the actin cytoskeleton in each cell type before infection. The virus strains may activate distinct signaling pathways that influence the ability of infected cells to remodel the actin cytoskeleton in a cell type-dependent manner. Additional experiments are necessary to examine these possibilities.

Interestingly, we also observed that pp65 was retained in the nuclei of TR-BAC infected THP-1 derived macrophages. In addition, the localization of pp65 to the cytoplasm was slow in Towne-infected THP-1 macrophages relative to primary fibroblasts. Casavant et al. reported a delay in the transition of pp65 to the cytoplasm in Towne-infected p53-null fibroblasts (Casavant et al., 2006), so it is possible that the delay in THP-1 macrophages can be explained the absence of functional p53 in these cells. Nevertheless, the strain-specific differences in pp65 localization we observed in THP-1 derived macrophages were also detected in primary fibroblasts. Nuclear export of pp65 from the nucleus is regulated by cyclin-dependent kinase (cdk) activity (Sanchez et al., 2007; Sanchez and Spector, 2006), which may suggest that the TR-BAC strain of virus does not activate cdks in the same manner as Towne.

Taken together, our work in THP-1 macrophages and fibroblasts has revealed novel strain-specific differences in the ability of HCMV to remodel the cytoskeleton and to regulate tegument protein localization. Thus, these studies validate the use of differentiated THP-1 cells as a model for lytic infection.

Materials and methods

Cell culture and virus

HCMV virus strains were propagated in primary human fibroblasts as previously described (Tamashiro et al., 1982). The laboratory strain Towne was obtained from American Type Culture Collection (ATCC; VR 977). The TR-BAC was prepared from the TR clinical HCMV isolate (a gift from Dr. Jay Nelson, Oregon Health Sciences University). The BAC clone was generated by substituting BAC DNA for the US2–US5 region of the TR genome (Murphy et al., 2003). It should be noted that stocks of virus were prepared in fibroblasts not endothelial cells as previously described (Sinzger et al., 2006).

THP-1 cells (ATCC) were maintained in RPMI Medium 1640 supplemented with 10% heat-inactivated fetal bovine serum,

glutamine, penicillin and streptomycin. Cells were counted, seeded into tissue culture plates, and differentiated 22–24 h before infection by treatment with phorbol myristate acetate (PMA; EMD Biosciences) at a final concentration of 50 ng/mL. Adherent cells were infected with HCMV Towne or TR-BAC at a multiplicity of infection (MOI) of 5 in the presence of PMA and hydrocortisone (HC; 5 μM; Sigma). Twenty-four h p.i., the virus inoculum was removed and cultures were fed with complete medium containing PMA and HC. Cells were harvested at various times p.i. and pellets were frozen until further analysis. Alternatively, supernatants containing progeny virions were collected at the times indicated and viral titers were determined on primary human fibroblasts as previously described (Tamashiro et al., 1982). For some experiments, cell pellets were collected along with supernatants for measurement of intracellular virus titer. Briefly, pelleted cells were resuspended in complete media and drawn through a sterile syringe with a 25-gauge needle. The lysate was gently sonicated in a water bath sonicator for 2 min then frozen. Viral titer was determined on primary human fibroblasts as described above.

Human foreskin fibroblasts (HFF) were maintained as previously described in minimal essential media (MEM)-Earle's media supplemented with 10% fetal bovine serum, penicillin, streptomycin, amphotericin, and glutamine (Sanchez et al., 2002). For G_0 infections, cells were grown to confluence allowed to arrest for 3 additional days before trypsinization and replating at a lower density (Sanchez et al., 2003). Cells grown on coverslips were infected at a MOI of 5 with Towne or TR-BAC the time of release from confluence. Mock-infected cells were incubated with an equal volume of conditioned media.

UV inactivation of virus

To determine the effects of the viral secretome on THP-1 cell morphology, the virus inoculum was inactivated by UV irradiation as previously described (Fortunato et al., 2000). An equivalent number of plaque forming units (PFU) Towne or TR-BAC stocks were diluted in RPMI and exposed to 6000 J/m^2 of irradiation in a Stratalinker. Sodium pyruvate (5 mM), PMA, and HC were added to the inoculum after UV treatment, which was then used to infect THP-1 derived macrophages 24 h post-differentiation as described above. Cells were infected with Towne or TR-BAC in parallel. At 48, 72, 96, and 120 h p.i., cells were visualized by phase contrast microscopy and images were recorded.

Western blotting

At the time points indicated, HCMV- and mock-infected cells were collected by trypsinization and frozen. Pellets were resuspended in reducing sample buffer (RSB) (Sanchez et al., 2002) and disrupted by passing samples several times through a 25-gauge needle. Samples were boiled for 3 min prior to loading. Equivalent cell numbers were loaded into each lane of polyacrylamide gels and proteins were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose. The following antibodies were used to probe filters: anti-IE1/IE2, anti-UL44, and anti-UL57 (Virusys); anti-IE1, anti-pp65, anti-pp28 (a gift from Dr. William Britt, University of Alabama, Birmingham); anti-IE2 (Chemicon); and anti-beta actin (Sigma).

Analysis of viral DNA replication by real time PCR

THP-1 derived macrophages were infected with Towne or TR-BAC at an MOI of 5 as described above. Cells were collected at 24, 48, 72, 96, 120, and 144 h p.i. and frozen. To measure viral DNA replication in fibroblasts, cells were infected as described above and infected with Towne at a MOI of 5. Cells were collected and pellets were frozen at the time points indicated. DNA was extracted from the pellets using the Qiagen Mini-Blood Kit per manufacturer's instructions. Viral DNA replication was measured by quantitative real time PCR using TaqMan One-Step PCR master mix reagents kit (Applied Biosystems) and primers to HCMV UL77 (White et al., 2004), TaqMan dual-labeled (5′ fluorescein (FAM) - 3′ Black Hole quencher) probes (Integrated DNA Technologies) as described previously (White et al., 2004). A standard curve was made using dilutions of the DNA from samples harvested at 24 h p.i. Fold differences were calculated relative to the level of viral DNA at 24 h p.i. Samples were normalized by measuring the amplification of the cellular beta actin gene as an internal standard. Primers and probe for beta actin were purchased from Applied Biosystems.

Immunofluorescence

THP-1 monocytes were seeded onto glass coverslips and treated with PMA to induced differentiation 22–24 h prior to infection Towne or TR-BAC at an MOI of 5 as described above. For infections in primary human fibroblasts, G_0-synchronized cells were seeded onto glass coverslips and infected at a MOI of 5. At the time points indicated, cells were rinsed with PBS and fixed in 2% paraformaldehyde in PBS for 15 min at room temperature. Cells were permeabilized and stained as previously described (Sanchez et al., 2002). Coverslips were stained with anti-pp65 antibody (a gift from Dr. William Britt); anti-UL44 antibody (Virusys); anti-GM130 and anti-LAP2 antibodies (BD Biosciences); and FITC-phalloidin (Sigma). Coverslips were mounted with SlowFade reagent (Invitrogen) and visualized using an Olympus IX81 research microscope equipped with a DSU spinning disk confocal unit. Images were captured by either epifluorescent or confocal imaging techniques as indicated in the figure legends. For measurement of the number of cells containing pp65 in nuclei versus cytoplasm, 5–10 images were recorded per coverslip and approximately 100 cells were counted per experimental condition. For quantitation of cells containing actin fibers, 8–18 images were recorded per coverslip (approximately 100 cells on average per experimental condition). Data from 2–4 independent experiments were analyzed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.07.015.

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